Antagonistic Effects of the Staphylococcal Enterotoxin A Mutant, $SEA_{F47A/D227A}$, on Psoriasis in the SCID-hu Xenogeneic Transplantation Model

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Psoriasis is a T-cell-mediated immune dermatosis probably triggered by bacterial superantigens. This pathomechanism has been experimentally reproduced in a SCID-hu xenogeneic transplantation model. We analyzed the effects of different bacterial superantigens on the induction of psoriasis in this model. Staphylococcal enterotoxin B and exfoliative toxin triggered the onset of psoriasis when administered repetitively intracutaneously over a period of 2 wk, whereas staphylococcal enterotoxin A representing a distinct subfamily of staphylococcal enterotoxins only mimicked certain aspects of psoriasis. The biologic effects of staphylococcal enterotoxin A were more pronounced when a mutated form, SEA_{H187A}, of this superantigen with reduced affinity to major histocompatibility complex class II was coinjected. Another mutated variant, $SEA_{F47A/D227A}$, exhibiting no measurable major histocompatibility complex class II affinity blocked the effects triggered by wild-type staphylococcal enterotoxin A when injected in a 10-fold higher dose. Inhibition was specific as induction of psoriasiform epidermal changes by staphylococcal enterotoxin B could not be blocked. As staphylococcal enterotoxin A, in contrast to the other superantigens tested, is capable of inducing epidermal thickening but not the typical appearance of psoriasis, we conclude that bacterial superantigens may differ with regard to their effects on human nonlesional psoriatic skin. Staphylococcal-enterotoxin-A-mediated effects were blocked by a genetically engineered superantigen highlighting the potential therapeutic use of mutated superantigens.

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Bacterial superantigens (Acha-Orbea, 1995) are characterized by their ability to interact with and activate T cells that share defined T cell receptor Vβ segments. The portion of the T cell repertoire activated by any given superantigen is several orders of magnitude higher than activation by conventional antigen and lies in the range of 10%. This response is not human leukocyte antigen (HLA) restricted because superantigens associate with HLA class II molecules outside the peptide binding groove. Different superantigens do exhibit distinct preferences regarding -DR, -DP and -DQ isotypes, however (Herrmann *et al*, 1989; Mollick *et al*, 1991).

Among bacterial superantigens the staphylococcal enterotoxins (SEs) (Svensson *et al*, 1997) are a family of structurally related exotoxin molecules produced by certain Gram-positive *Staphylococcus aureus* bacterial strains. Comparison of sequence homologies allows division of the SEs into two subfamilies: SEB and SEC_{1-3} have marked homology, forming one subfamily, whereas SEA, SED, SEE, and SEH form a second subfamily. TSST-1 is another superantigen secreted by *S. aureus* exhibiting structural relationships to the SE family. Finally, approximately 5% of *S. aureus* strains secrete staphylococcal exfoliative toxins (ETs), designated serotypes A and B. ETA, which is the causative agent in staphylococcal scaled skin syndrome, functions as serine protease, but also has superantigen properties (Vath *et al*, 1997).

There is increasing evidence that superantigens are involved in the pathogenesis of several autoimmune diseases, e.g., rheumatoid arthritis (Paliard *et al*, 1991) and diabetes mellitus (Conrad *et al*, 1994). Another T-cell-mediated autoimmune disease is psoriasis. Clinically, there is an association of this disease with bacterial infections, an observation that led to the hypothesis of psoriasis being triggered by superantigen-activated T cells cross-reacting with keratins (Valdimarsson *et al*, 1995). Induction of psoriasis could be demonstrated experimentally in the SCID-hu xenogeneic transplantation model by injecting bacterial superantigens into nonlesional psoriatic skin transplanted onto mice lacking functional B and T cells (Boehncke *et al*, 1996). This phenomenon was found to be T cell dependent (Wrone-Smith and Nickoloff, 1996).

Based on the potential of SEs to induce psoriasis we investigated the possibility of interfering with this process by applying mutated forms of the respective SEs. Here we demonstrate that a mutation of F47 to A and D227 to A in SEA causing loss of major histocompatibility complex (MHC) class II affinity specifically inhibits the effects triggered by wild-type SEA when injected in a 10-fold higher dose.

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Abbrevations: ET, exfoliative toxin; PBMC, peripheral blood mononuclear cells; SE, staphylococcal enterotoxin.

MATERIALS AND METHODS

Patients This study was approved by the ethics committee of the Faculty of Medicine of the Johann-Wolfgang-Goethe University, Frankfurt. Written informed consent was obtained from nine patients with chronic plaque-stage psoriasis and nonlesional skin was excised from the inner aspect of the upper arm under local anesthesia.

Transplantation procedure The animal experiments were approved by the Regierungspräsidium Darmstadt (II17a–19c 20/15–F 79/02). Transplantations were done as described previously (Boehncke *et al*, 1994). Human full-thickness xenografts were transplanted onto the back of 6–8-wk-old C.B17 SCID mice (Charles River, Sulzfeld, Germany). For the surgical procedure, mice were anesthetized by intraperitoneal injection of 100 mg per kg ketamine and 5 mg per kg xylazine. Spindle-shaped pieces of full-thickness skin measuring 1 cm in diameter were grafted onto corresponding excisional full-thickness defects of the shaved central dorsum of the mice and fixed by 6-0 atraumatic monofilament sutures. After applying a sterile Vaseline-impregnated gauze, the grafts were protected from injury by suturing a skin pouch over the transplanted area using the adjacent lateral skin. The sutures and over-tied pouches were left in place until they resolved spontaneously after 2–3 wk.

Treatment protocols Grafts were allowed 4 wk for acceptance and healing onto the mice. Injections were performed on days 28, 31, 34, and 37 after transplantation.

For analysis of the biologic effects of bacterial superantigens, 2 μg of either ET (Toxin Technologies) or SEA or SEB (Active Biotech, Lund, Sweden) representing different SE subfamilies were injected intradermally in a final volume of 200 μl. In parallel, 2×10^6 of the donors' peripheral blood mononuclear cells (PBMC) were injected intraperitoneally in 100 μl phosphate-buffered saline (PBS). These cells were prepared from peripheral blood taken at the time of skin excision by Ficoll density gradient sedimentation (Sigma, Germany), frozen in medium containing 90% fetal bovine serum (FBS) and 10% dimethylsulfoxide, and stored at –80°C. Forty-eight hours prior to injection the PBMC were thawed and cultured at 37°C in an atmosphere containing 5% CO_2. The medium used was supplemented RPMI-1640 (Seromed, Berlin, Germany). Cells were stimulated for the total period of 48 h with the corresponding superantigens at a concentration of 100 ng per ml. PBS served as negative control both *in vitro* and *in vivo*.

To assess the capability of mutant superantigens to block the effects induced by wild-type superantigens the former were added to the cell cultures *in vitro* 2 h prior to the latter and also injected into the corresponding grafts at a 10-fold excess (1 μg per ml *in vitro* and 20 μg *in vivo*, respectively).

In all experiments three grafts of three different donors underwent an identical treatment protocol. Thus, the data presented are based on findings of nine different grafts.

For the analysis of antibody production towards wild-type SEA and $SEA_{F47A/D227A}$ two mice each were injected intraperitoneally with PBMC stimulated by wild-type SEA either alone or in combination with $SEA_{F47A/D227A}$ followed by intramuscular injections twice weekly for 2 wk. Serum was analyzed for the presence of anti-SEA antibodies in an enzyme-linked immunosorbent assay.

***In vitro* mutagenesis and protein production** *In vitro* mutagenesis of genetically engineered SEA variants, protein expression, and purification were performed as described previously (Abrahmsen *et al*, 1995).

Analysis of the grafts Mice were sacrificed at day 40 and following excision with surrounding mouse skin the grafts were formalin-embedded. Subsequently, routine hematoxylin and eosin stainings were performed and the grafts were analyzed with regard to their pathologic changes both qualitatively (epidermal differentiation, inflammatory infiltrate) and quantitatively (epidermal thickness) by a blinded investigator as described previously (Boehncke *et al*, 1994). Briefly, maximal epidermal thickness was measured from the tip of the rete ridges to the border of the viable epidermis. The values were determined using an ocular micrometer, taking the mean of 10 consecutively measured rete ridges. The results were expressed as mean ± standard deviation in microns. Statistical analyses were performed using Graph Pad Instat software and the paired nonparametric Kruskal–Wallis test calculating one-tail p-values.

Antagonistic effects of SEA_{wt} and $SEA_{F47A/D227A}$ *in vitro* Cytotoxicity was measured in a 4 h ^{51}Cr release assay (Rosendahl *et al*, 1999). An SEA-reactive human T cell line was used as effector cells and was preincubated for 2 h with varying concentrations of $SEA_{F47A/D227A}$. SEA_{wt} was added at varying concentrations together with ^{51}Cr-labeled human MHC class II expressing Raji cells. The mixtures were cultured with 2500 target cells per 0.2 ml complete R-medium (RPMI 1640; BioWhittaker, Verviers, Belgium) supplemented with 10% FBS (HyCrone, Logan, UT), 5×10^{-5} M β-mercaptoethanol (Merck, Darmstadt, Germany), and 0.1 mg per ml gentamycin (Biological Industries, Beit Haemek, Israel) at an E:T ratio of 30:1. The percentage of specific cytotoxicity was calculated as 100 × (cpm experimental release − cpm background release)/(cpm total release − cpm background release).

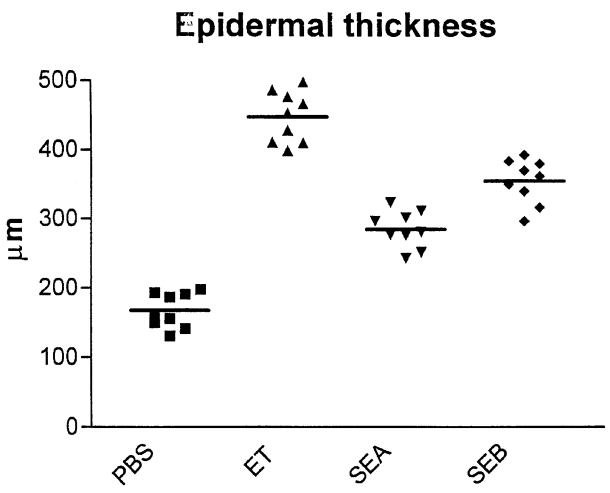

Figure 1. Epidermal thickness of human nonlesional psoriatic skin transplanted onto SCID mice. The line marks the mean. ET and SEB induce statistically significant akanthosis, whereas SEA induces significantly less epidermal thickening compared with ET ($p < 0.01$).

RESULTS

Different staphylococcal superantigens exhibit distinct effects in nonlesional psoriatic skin The biologic effects of different bacterial superantigens were analyzed in nonlesional skin grafts from three patients with chronic plaque-stage psoriasis.

ET has previously been shown to be capable of inducing psoriasiform changes in nonlesional psoriatic skin grafted onto SCID mice (Boehncke *et al*, 1996) and was therefore selected as positive control. Repetitive intradermal injections with ET along with intraperitoneal injections of autologous PBMC stimulated *in vitro* with ET yielded statistically significant akanthosis (448 ± 37 μm, $p < 0.001$, **Fig 1**, **Table I**) and papillomatosis along with parakeratosis and reduction or even complete loss of the granular layer. Moreover, a mononuclear infiltrate occurred in the upper dermis exhibiting some exocytosis, but Munro's micro-abscesses were not detected (**Table I**, **Fig 2*c*, *d***).

SEA and SEB were selected as representatives of the two major SE subfamilies. Injections with SEA and SEA-activated PBMC resulted in obvious akanthosis, which was significantly less pronounced compared with ET ($p < 0.01$, **Fig 1**, **Table I**). Papillomatosis was not seen, but parakeratosis was a constant feature. Munro's microabscesses were occasionally seen (**Table I**, **Fig 2*e*, *f***). SEB effects were characterized by induction of a more pronounced akanthosis and papillomatosis compared with SEA and an inflammatory infiltrate characterized by the frequent occurrence of Munro's microabscesses (**Table I**, **Figs 1**, **2*g*, 2*h***). Both SEA and SEB caused comparable reduction of the granular layer.

Mutations in the HLA class-II binding site of SEA modulate the *in vivo* activity of SEA In order to interfere with the biologic effects exhibited by the superantigens included in this study mutations of wild- type SEA were generated and tested in nonlesional skin grafts derived from three patients suffering from

Table I. Biologic effects of several staphylococcal superantigens on human nonlesional psoriatic skin

Protocol	Epidermal thickness[a]	Papillomatosis	Keratinization	Infiltrate	Comments
PBS	167 ± 25	–	orthokeratosis	sparse	negative control
ET	448 ± 37*	++	parakeratosis	intermediate	positive control loss of granular layer
SEA	285 ± 27**	–	parakeratosis	intermediate	occasionally Munro's reduction of granular layer microabscesses
SEB	355 ± 32***	++	parakeratosis	dense	frequently Munro's reduction of granular layer microabscesses

[a]Mean and standard deviation in μm; *$p < 0.001$ *versus* PBS; **$p < 0.01$ *versus* ET; ***$p < 0.01$ *versus* PBS.

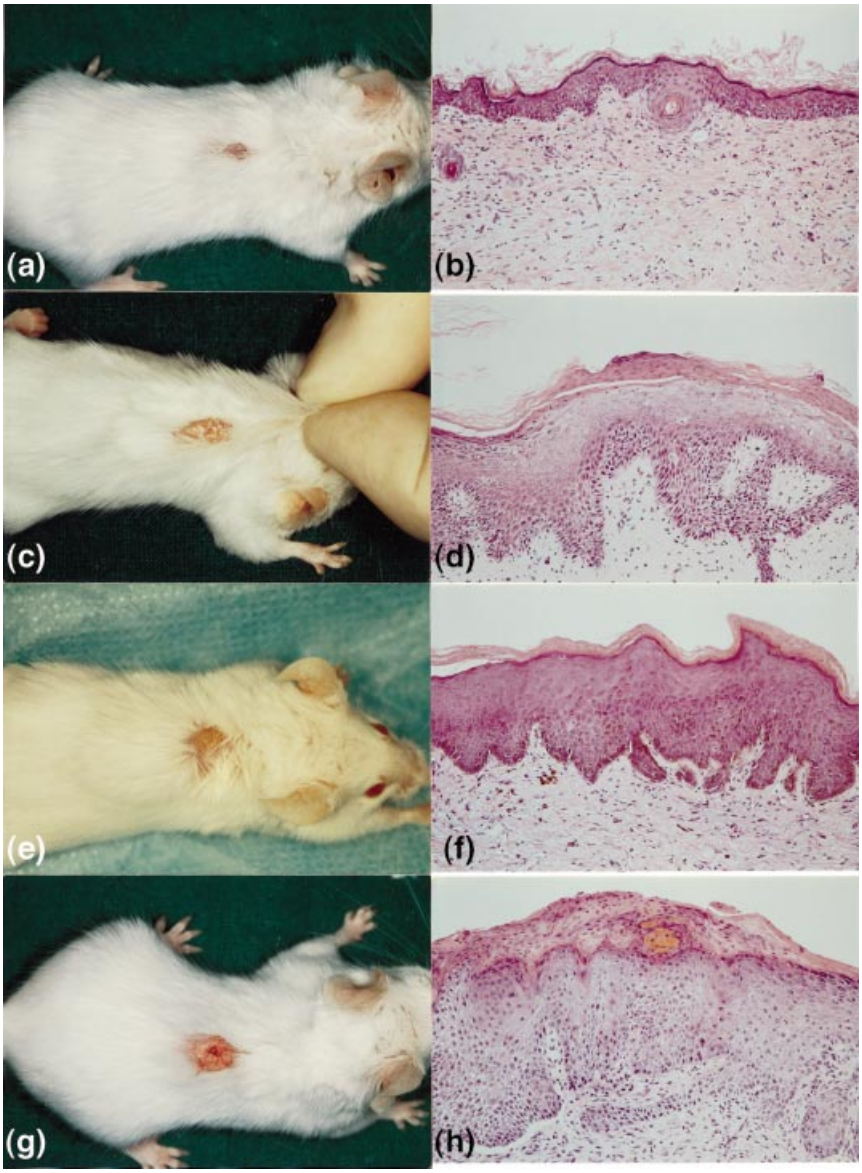

Figure 2. Macroscopic and histologic appearance of human nonlesional psoriatic skin transplanted onto SCID mice. PBS-treated controls remain largely unaltered (*a*, *b*), whereas grafts receiving wild-type SEs show different degrees of psoriasiform transformation. ET-treated grafts appear thicker and erythematous (*c*); histologically akanthosis, papillomatosis, and orthokeratosis along with an exocytotic infiltrate are observed (*d*). SEA-treated grafts lack papillomatosis (*e*, *f*), whereas grafts challenged with SEB (*g*, *h*) frequently exhibit Munro's microabscesses.

chronic plaque-stage psoriasis (not identical with the patients in the first set of experiments).

Alanine substitution of a histidine at position 187 in SEA (SEA_{H187A}) results in an approximately 10-fold reduction in affinity towards MHC class II (Abrahmsen *et al*, 1995). This mutant is able to induce some akanthosis when injected at doses of 20 μg into nonlesional psoriatic skin (**Table II**). Combination with SEA_{wt} resulted in an additive effect with respect to akanthosis (**Fig 3**). Moreover, papillomatosis was noted (**Table II**, **Fig 4*a*, *b***). This feature is not easily explained as an additive effect as the effect was not present in grafts treated with either SEA_{wt} or SEA_{H187A} alone.

Another mutated peptide designated $SEA_{F47A/D227A}$ exhibiting no measurable MHC class II affinity did not exhibit any effects at doses of 20 μg on nonlesional psoriatic skin (**Table II**, **Figs 3**, **4*c*, 4*d***). Most interestingly it was capable of inhibiting the effects of SEA_{wt}: grafts pretreated with 20 μg $SEA_{F47A/D227A}$ and subse-

Table II. Biologic effects of two genetically engineered SEA variants on human nonlesional psoriatic skin

Protocol	Epidermal thickness[a]	Papillomatosis	Keratinization	Infiltrate	Macroscopic appearance
SEA_{H187A}	250 ± 29	–	orthokeratosis	sparse	largely unaltered
$SEA_{F47A/D227A}$	157 ± 48	–	orthokeratosis	sparse	largely unaltered
SEA_{wt} + SEA_{H187A}	291 ± 29*	+	parakeratosis	dense	thickened, erythematous
SEA_{wt} + $SEA_{F47A/D227A}$	182 ± 31**	–	orthokeratosis	moderate	slightly thickened and erythematous
SEB_{wt} + $SEA_{F47A/D227A}$	313 ± 32***	++	parakeratosis	dense	profound thickening intermediate erythema

[a]Mean and standard deviation in μm; *$p < 0.001$ *versus* $SEA_{F47A/D227A}$; **$p < 0.01$ *versus* SEA_{wt} + SEA_{H187A}; ***$p < 0.001$ *versus* SEA_{wt} + $SEA_{F47A/D227}$ and $p < 0.001$ *versus* $SEA_{F47A/D227A}$.

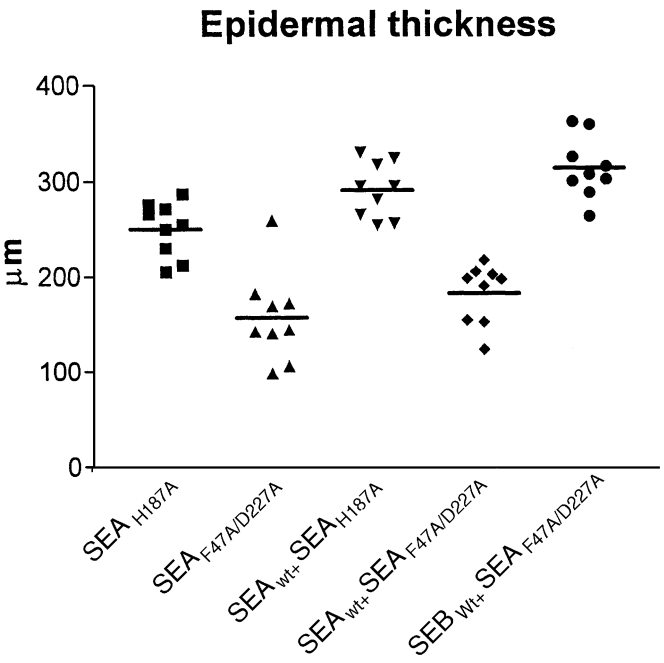

Figure 3. Epidermal thickness of human nonlesional psoriatic skin transplanted onto SCID mice. The line marks the mean. The SEA mutant $SEA_{F47A/D227A}$ blocks induction of akanthosis by the wild-type SEA significantly ($p < 0.01$), whereas epidermal thickening induced by SEB is not affected by this protein.

quently challenged with SEA_{wt} at a dose of 2 μg were characterized by only minimal akanthosis; the degree of akanthosis was significantly reduced compared to grafts receiving SEA_{wt} along with the nonblocking mutant SEA_{H187A} ($p < 0.01$). Signs of abnormal keratinization were also minimal, usually a granular layer was established, and the corneal layer frequently exhibited the normal web-like structure (**Table II**, **Fig 4*e*, *f***). Thus, $SEA_{F47A/D227A}$ seems to exhibit antagonistic effects on SEA_{wt} in this model.

These differences between grafts treated with SEA in the presence or absence of SEA_{H187A} or $SEA_{F47A/D227A}$ were visible already macroscopically: SEA_{wt}-treated grafts appeared thicker and erythematous compared with PBS-treated controls (**Fig 2*a*, *b*** *versus* **Fig 2*e*, *f***). These changes were more pronounced when SEA_{wt} and SEA_{H187A} were coadministered (**Fig 4*a*, *b***). In contrast, grafts treated with wild-type SEA_{wt} and $SEA_{F47A/D227A}$ appeared to be only slightly thicker and more erythematous than PBS-treated controls (**Fig 4*e*, *f***).

The antagonistic effect of $SEA_{F47A/D227A}$ is not due to antibody formation A possible explanation for the blocking effect of $SEA_{F47A/D227A}$ could be the induction of neutralizing human anti-SEA antibodies. To test this possibility two mice each were injected intraperitoneally with PBMC stimulated by SEA_{wt} either alone or in combination with $SEA_{F47A/D227A}$ followed by intramuscular injections twice weekly for 2 wk. The presence of human anti-SEA antibodies was below the limit of detection for the assay (i.e., 0.8 μg per ml) in all samples.

The antagonistic effect of $SEA_{F47A/D227A}$ is specific In order to test whether $SEA_{F47A/D227A}$ might represent a more general superantigen inhibitor nonlesional skin grafts from three psoriatic patients (not identical with those in the first two sets of experiments) were pretreated with $SEA_{F47A/D227A}$ and then challenged with SEB_{wt}. In these experiments the phenotype induced by SEB_{wt} alone characterized by extensive akanthosis and papillomatosis could not be altered (**Table II**, **Figs 3**, **4*g*, *h***). Thus, $SEA_{F47A/D227A}$ seems to be capable of specifically interfering with the effects of SEA *in vivo*.

$SEA_{F47A/D227A}$ inhibits SEA_{wt}-mediated effects *in vitro* In order to analyze *in vitro* inhibition of SEA_{wt}-mediated effects of $SEA_{F47A/D227A}$ cytotoxicity assays were performed using an SEA-reactive cytotoxic human T cell line as effector cells together with ^{51}Cr-labeled human HLA class II expressing Raji cells. Incubation with SEA_{wt} resulted in >50% cytotoxicity at concentrations $>10^{-12}$ M, whereas no measurable cytotoxicity occurred with $SEA_{F47A/D227A}$ at concentrations up to 10^{-9} M (**Fig 5**). Pre-incubation of Raji cells with $SEA_{F47A/D227A}$ and subsequent addition of SEA_{wt} resulted in a dose-dependent reduction of cytotoxicity (**Fig 5**) thus documenting the *in vitro* inhibitory efficacy of $SEA_{F47A/D227A}$.

DISCUSSION

Using the SCID-hu xenogeneic transplantation model it was possible to directly demonstrate induction of psoriasis by bacterial superantigens (Boehncke *et al*, 1996; Wrone-Smith and Nickoloff, 1996). Here we highlight the possibility of specifically interfering with this process by applying mutant forms of the respective wild-type superantigen.

The SCID-hu xenogeneic transplantation system is now a widely accepted model for studying the pathophysiology of complex human diseases in general (Boehncke, 1999) and of psoriasis in particular (Schön, 1999). Recently the suitability of the model for the screening of potential antipsoriatic treatments was demonstrated (Boehncke *et al*, 1999; Dam *et al*, 1999). Therefore, this system was chosen to analyze the biologic effects of superantigens in human skin.

Regarding the biologic effects of bacterial superantigens two scenarios are possible: the effects seen either could be intrinsic properties of the respective superantigens, or they could depend on the environment. The former point of view is supported by evidence for the involvement of staphylococcal superantigens in the pathogenesis of atopic dermatitis (Leung, 1997), whereas streptococcal superantigens are thought to be more relevant for the pathogenesis of psoriasis (Valdimarsson *et al*, 1995). Whether this association is indeed so strict is still a matter of debate, as at least with reference to psoriasis reports on the presence of a T cell infiltrate primarily responsive to streptococcal superantigens (Baker *et al*, 1995; Leung *et al*, 1995) stand beside findings documenting T

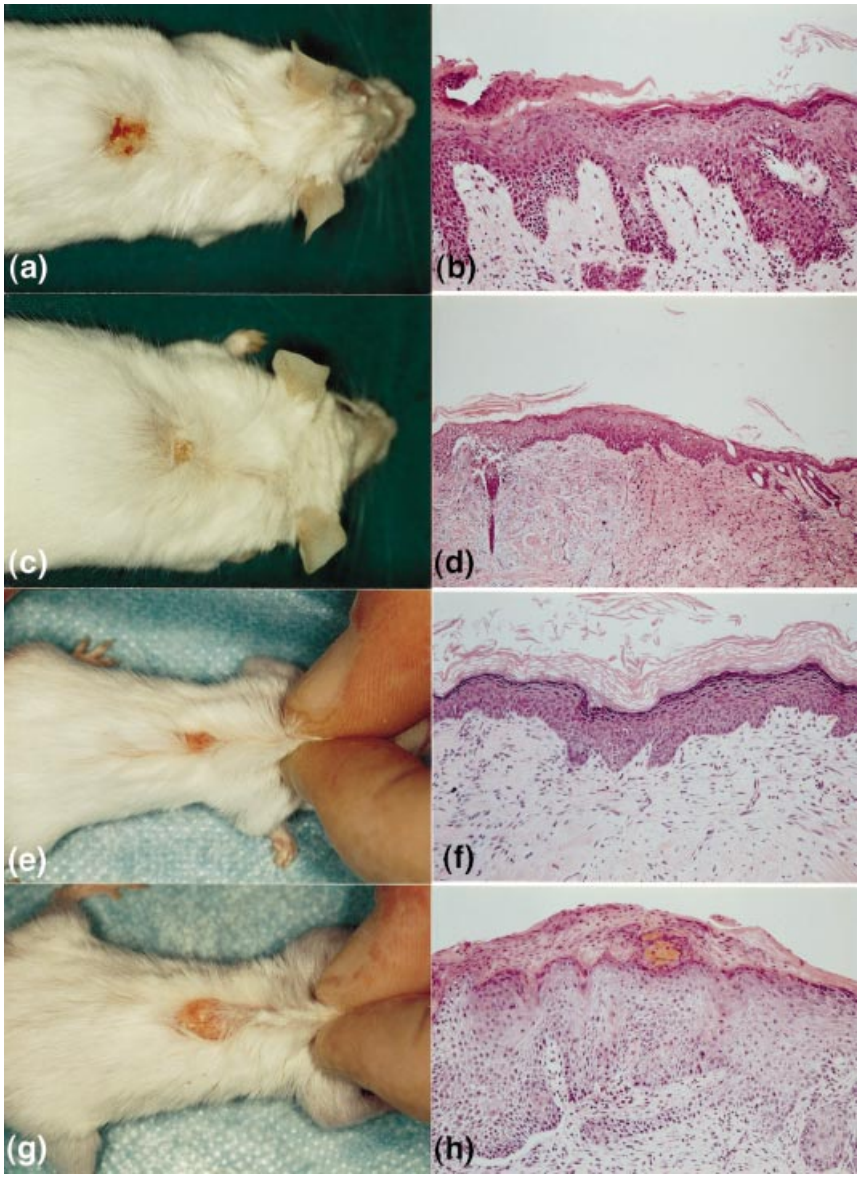

Figure 4. Effects of genetically engineered SEA variants on human nonlesional psoriatic skin treated with wild-type SEs. SEA_{wt} and SEA_{H187A} exhibit additive effects and also show papillomatosis (*a*, *b*). $SEA_{F47A/D227A}$ alone does not alter the grafts (*c*, *d*) but is capable of blocking SEA_{wt}-mediated transformations (*e*, *f*). Changes induced by SEB_{wt}, however, are not affected (*g*, *h*).

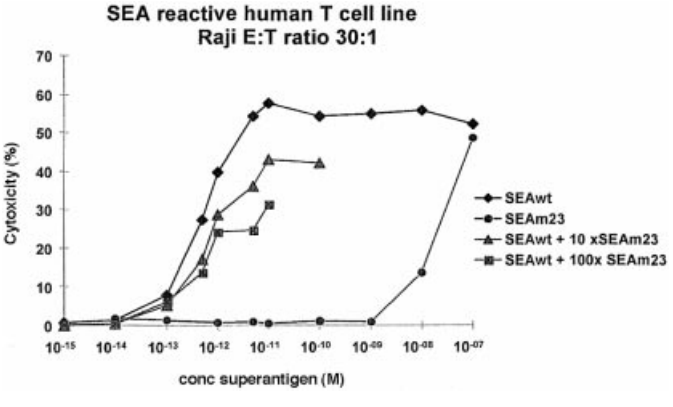

Figure 5. The ability of $SEA_{F47A/D227A}$ to inhibit SEA-dependent cellular cytotoxicity against MHC class II^+ Raji cells was analyzed in a 4 h ^{51}Cr release assay. SEA-stimulated human T cell line was used as effector cells. Data from one of three representative experiments are shown.

cells reacting towards staphylococcal superantigens (Bour *et al*, 1995; Yokote *et al*, 1995). Moreover, in the SCID-hu model applied also in this study we and others were able to demonstrate inducibility of psoriasis by staphylococcal superantigens (Boehncke *et al*, 1996; Wrone-Smith and Nickoloff, 1996). The latter reports also document lack of induction of inflammatory alterations in grafts derived from normal human skin by the respective superantigens. Thus, superantigens alone are not sufficient to trigger psoriasis, and the ''source'' of the graft has an impact on their effects. In this report we observed that superantigens representing distinct SE subfamilies differ with regard to their effects on grafts derived from nonlesional skin of psoriatic patients, although they all caused changes that resembled each other. Therefore we think that a predisposition intrinsic to the skin compartment is needed in order to induce psoriasis in nonlesional skin. On the other hand the ''psoriatrogenic'' efficacy of distinct SEs differs markedly.

Superantigens seem to play a crucial role in the development of T-cell-mediated autoimmunity. Although potentially autoreactive T cells are eliminated in the thymus by means of negative selection/depletion a small fraction of those cells can still be found, but the respective cells are in the state of anergy (Nossal, 1994). Anergy can be broken when both the suitable autoantigen and a costimulatory signal are present simultaneously. This cascade of events can be demonstrated in animal models using a bacterial superantigen as stimulus (Brocke *et al*, 1993). Besides psoriasis (Valdimarsson *et al*, 1995; Boehncke, 1996), there is evidence for the involvement of

superantigens also in other human diseases (Paliard *et al*, 1991; Conrad *et al*, 1994). It is therefore intriguing to speculate about the possibility of interfering with the superantigen-mediated induction of autoimmunity. In this report we were able to demonstrate interference with the SEA-mediated induction of psoriasis by means of a mutated form of the respective superantigen. As $SEA_{F47A/D227A}$ lacks measurable MHC class II affinity the inhibitory effect on SEA_{wt} could be due to competition for available T cell receptor Vβ elements. Alternatively, binding of the nonfunctional $SEA_{F47A/D227A}$ mutant could result in a nonactivating downregulation of the T cell receptor (Valltutti *et al*, 1995). Given the distinct repertoire of T cell receptor Vβ elements recognized by any given SE, we interpret the specificity of the inhibition of $SEA_{F47A/D227A}$, which blocks effects in nonlesional psoriatic skin mediated by SEA_{wt} but not SEB_{wt}, as further evidence for $SEA_{F47A/D227A}$ acting at the level of the T cell receptor. The possibility of induction of anti-SEA antibodies by the mutated $SEA_{F47A/D227A}$ was ruled out, as no such antibodies could be detected in the sera of the mice.

In the field of oncology, SEA application for therapeutic purposes has already been undertaken in the form of fusion proteins composed of a tumor-reactive monoclonal antibody and the superantigen (Giantonio *et al*, 1997; Gidlöf *et al*, 1997; Hansson *et al*, 1997). Here, too, alteration of MHC class II affinity was necessary to yield a practical therapeutic tool: reducing the MHC class II affinity of SEA resulted in reduced systemic immune activation and thus toxicity of this approach (Hansson *et al*, 1997). For psoriasis, two possible – highly speculative at this point in time, however – clinical applications could be imagined. Frequently, a new rash of an already established psoriasis is triggered by infections with superantigen-producing bacteria; these infections precede the rash by several weeks. Identification of the relevant superantigen and subsequent vaccination with a mutated form of the respective superantigen might prevent the rash. A prophylactic vaccination with genetically engineered superantigens of patients at risk of developing psoriasis would be even more beneficial. Right now, at least the population at risk of developing psoriasis could principally be defined in part: people with a positive family history exhibiting certain HLA class II genotypes have a dramatically higher risk of developing psoriasis (Christophers and Henseler, 1989; Tomfohrde *et al*, 1994; Burden *et al*, 1998). Application of this potential vaccination strategy would be limited, however, to those cases of psoriasis where the disease or the respective rash is triggered by infection with superantigen-producing bacteria. The remainder of the patients would not be expected to benefit from this approach.

In summary, this report documents differences regarding the ''psoriatrogenic'' efficacy of SEs representing distinct subfamilies in nonlesional psoriatic skin. Triggering psoriasis in this system can specifically be blocked by a mutant SE lacking measurable MHC class II binding affinity, thus indicating the potential therapeutic use of altered bacterial superantigens.

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